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(54) Title: EPIDERMAL GROWTH FACTOR RECEPTOR ECTODOMAIN (57) Abstract Diagnostic/prognostic methods are provided for detecting and/or quantitating in the body fluids, preferably serum, of mammals carrying a malignant tumor burden, elevated levels of a portion of the epidermal growth factor receptor (EGFr) which comprises substantially the EGFr ectodomain having a molecular weight in the range of from about 90 kilodaltons (kd) to about 115 kd, preferably from about 95 kd to about 105 kd, and more preferably about 100 kd. Substantially pure compositions comprising said EGFr ectodomain protein and/or fragments thereof are disclosed as well as test kits for performing the disclosed assays.		

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EPIDERMAL GROWTH FACTOR
RECEPTOR ECTODOMAIN

FIELD OF THE INVENTION

This invention is in the medical arts; more specifically, it is in the area of diagnostics/prognostics; and still more specifically, it is in the fields of oncology and immunochemistry. This invention relates to the epidermal growth factor receptor (EGFr) and assays designed to detect and/or quantitate in the body fluids of mammals a portion of said receptor, which comprises substantially the ectodomain of EGFr and has a molecular weight in the range of from about 90 kilodaltons (kd) to about 115 kd, preferably from about 95 kd to about 105 kd, and still more preferably about 100 kd.

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BACKGROUND OF THE INVENTION

Epidermal growth factor (EGF) in vitro can trigger a variety of morphological and biochemical changes that resemble those characteristic of transformed cells, and has also been implicated in the abnormal regulation of proliferation shown by transformed and tumor-derived cell lines. EGF delivers its signals by binding to specific protein receptors embedded in the cell's plasma membrane.

The receptor protein for EGF, the epidermal growth factor receptor (EGFr), is a 170 kilodalton (kd) transmembrane glycoprotein which comprises a cytoplasmic or tyrosine kinase domain, a transmembrane region and an extracellular domain or ectodomain which contains the binding site for epidermal growth factor (EGF) and transforming growth factor alpha (TGF- α) [Marquardt and Todaro, J. Biol. Chem., 257: 5220-5225 (1982); Carpenter et al., PNAS (USA) 80: 5627-5630 (1983)]. The cytoplasmic domain comprises 542 amino acids (C-terminal residues), and the ectodomain comprises 621 amino acids (N-terminal residues); they are linked by a short transmembrane region of about 23 amino acids [Ullrich et al., Nature, 309 (1983):

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418-425 (May 31, 1984); Gullick et al., Proc. R. Soc. Lond., B 226: 127-134 (1985)].

EGFr is a cell membrane macromolecule that is widely distributed in cells derived from all three embryonic germ layers. The wide distribution of the receptor in cells and tissues indicates that EGF may have an important role to play in growth control. Many normal cells express 10-100,000 EGFrS [Adamson and Rees, Mol. Cell Biochem., 34: 129-152 (1981); however, increased numbers of EGFrS are present in several types of human tumors, including gliomas and meningiomas, squamous carcinoma of the lungs, and ovarian, cervical and renal carcinomas [Thompson and Gill, Cancer Surveys, 4(4): 768-788 (1985)]. The human epidermoid carcinoma cell line A431 expresses about 10- to 50-fold more receptors than the majority of other cells [Fabricant et al., PNAS (USA), 74(2): 565-569 (1977); Wrann and Fox, J. Biol. Chem., 254: 8083-8086 (1979)]. Overexpression of EGFrS, as occurs in A431 cells, can augment cell growth because of increased formation of active ligand/receptor complexes.

Gene amplification has been demonstrated to be one mechanism for aberrant EGFr expression in A431 cells and in some glioblastoma multiforme tumors but not in all human tumors [Thompson and Gill, Cancer Surveys, 4(4): 768-788 (1985)]. Studies with tumor cell lines in vitro have shown a direct relationship between the levels of activated EGFr and cellular proliferation. Further, the growth rate of tumors in nude mice was shown to be directly related to the EGFr levels, that is, the most rapidly growing tumors displayed elevated EGFr levels [Thompson and Gill, id.].

EGF receptors (EGFrS) can be detected in a variety of cells either by measurement of EGF binding [reviewed in Adamson and Rees, Mol. Cell Biochem., 34:129-152 (1981)], by cross-linking of labeled EGF to its receptor [reviewed in Linsley et al., in Membrane Receptors (eds. Jacobs and Cuatrecasas), vol. B11: 87-113 (Chapman J. Hall, London and New York 1981)], or through the use of monoclonal antibodies [Schreiber et al., PNAS (USA), 78: 7535-7539 (1981);

Waterfield et al., J. Cell Biochem., 20: 149-161 (1982); Kawamoto et al., PNAS (USA), 80: 1337-1341 (1983); Richert et al., J. Biol. Chem., 258: 8902-8907 (1983); Gregoriou and Rees, Cell Biol. Int. Reports, 7: 539-540 (1983); and
5 Schlessinger et al., in Receptors and recognition: antibodies against receptors (ed. Greaves) (Chapman and Hall, London, 1985)].

Several groups have investigated the expression of EGFr in a variety of tumors using quantitative as well as
10 semiquantitative immunohistochemical methods. The types of tumors investigated include gynecological [Bauknecht et al., Gynecologic Oncology, 29(2): 147-157 (Feb. 1988); Gullick et al., Cancer Res., 46: 285-292 (1986)]; lung [Berger et al., J. Pathol., 152: 297-307 (1987)]; bladder [Smith et
15 al., Cancer Res., 49: 5810-5815 (1989); Neal et al., Lancet, 366-368 (1985)]; and breast carcinomas [Fitzpatrick et al., Cancer Res., 44: 3448-3453 (1984); Nicholson et al., Int. J. Cancer, 42: 36-41 (1988); and Nicholson et al., Lancet, 182-185 (1989)]. Such studies almost exclusively
20 rely upon radioligand binding methodology for quantitating EGFr in tissue samples.

The most extensive correlations of EGFr expression with clinical data have been carried out in studies with breast cancer patients [Nicholson et al., Int. J. Cancer,
25 42: 36-41 (1988); Sainsbury et al., Lancet, 1398-1402 (1987)]. In several studies with up to 246 patients, it was demonstrated that EGFr is a highly significant marker of poor prognosis for breast cancer [Sainsbury et al., id.]. It is considered to be the most important variable in
30 predicting relapse-free and overall survival in lymph node-negative patients, and to be the second most important variable, after nodal status, in lymph node-positive patients. In general, EGFr positive tumors are larger and occur in a higher proportion of patients with lymph node
35 involvement.

Further, in breast cancer studies, EGFr has been determined to be as good an indicator as the estrogen receptor (ER) in predicting the response to endocrine

therapy [Nicholson et al., Lancet, 182-185 (1989)]. Many ER positive patients don't respond to endocrine therapy, whereas a small percentage of ER negative patients do respond. EGFr positive tumors do not respond to endocrine therapy upon relapse, regardless of ER status, and EGFr positivity reduces the response rate even in ER positive tumors.

Studies have demonstrated a survival advantage for women that have ER positive tumors; most of such tumors are EGFr negative. ER negative tumors are divided into EGFr positive and negative groups, wherein the ER negative/EGFr positive group have a poor prognosis, and the ER negative/EGFr negative group have as good a relapse-free survival rate as the ER positive group. Thus, the measurement of EGFr levels is a useful adjunct to ER level measurement in breast cancer management.

Ovarian, cervical, vulval and endometrial tumors also overexpress EGFr. An association between EGFr levels and prognosis similar to that for breast cancer has been shown for endometrial cancer; however, increased EGFr levels associated with ovarian cancer correlate with a high response rate to chemotherapy [Bauknecht et al., supra]. EGFr expression and gene amplification has been more frequently observed among squamous cell carcinomas of the cervix and lung than in other types of cancer; however, correlation with tumor aggressiveness in such cancers has not been proven [Gullick et al., supra]. Quantitative studies with bladder tumors have shown that elevated EGFr levels are indicative of the most invasive tumors [Smith et al., supra; Neal et al., supra].

Ullrich et al., Nature, 309 (5967):418-425 (May 31, 1984), provides the complete 1,210 amino acid sequence of the EGFr precursor deduced from cDNA clones derived from placental and A431 carcinoma cells. Ullrich et al. note at page 425 that since the A431 cell line and the many variants thereof have "a multitude of defects exemplified by its 78 chromosomes, it is clearly best to regard this cell line with caution when exploring the mechanism of action of EGF

or in characterizing changes which relate in a meaningful way to neoplasia."

Weber and Gill, Science, 224: 294 (1984), reported that human epidermoid carcinoma A431 cells in culture produce a soluble 105 kilodalton (kd) protein which they determined to be related to the cell surface domain of the EGFr. See also, Weber, "Truncated EGF receptor: functional aspects of its secretion," [Acta Endocrinologica, 117 (Suppl. 287): 47 (No. 55) (1988)].

As indicated above, several methods have been used to detect EGFr levels in tumor tissues. There are, however, many cases in which tissue is not readily available or in which it is not desirable or not possible to withdraw tissue from tumors. Therefore, there is a need in the medical art for rapid, accurate diagnostic tests that are convenient and non-traumatic to patients. The invention disclosed herein meets said need by providing for non-invasive diagnostic/prognostic assays to detect and/or quantitate in mammalian body fluids, preferably serum, a portion of the EGFr which comprises substantially the ectodomain of EGFr.

SUMMARY OF THE INVENTION

Diagnostic/prognostic methods for neoplastic disease are provided, as well as, compositions and test kits for implementing such methods. The invention claimed herein is based upon the detection of a portion of the EGFr at elevated levels in the body fluids of mammals carrying a malignant tumor burden. Said portion of the EGFr comprises substantially the EGFr ectodomain. The inventors hereof show that one end of said EGFr portion has an amino acid sequence which is substantially identical to the N-terminus of the EGFr ectodomain [Ullrich et al., Nature, 309 (5967): 418-425 (May 31, 1984)]. Said EGFr portion, which is found in mammalian body fluids and has a molecular weight in the range of from about 90 kilodaltons (kd) to about 115 kilodaltons (kd), preferably in a range of from about 95 kd to about 105 kd, and more preferably in a range of about 100 kd, is herein termed "the EGFr ctodomain protein". Methods

and compositions are provided for detecting and/or quantitating said EGFr ectodomain protein.

Further, diagnostic/prognostic methods are provided wherein fragments of said EGFr ectodomain protein
5 are found in mammalian body fluids. For example, proteolytic activity in serum or in the kidneys may break down the EGFr ectodomain protein into fragments. Thus, for example, in serum or in urine such fragments which contain one or more epitopes of said EGFr ectodomain protein may be
10 detected and/or quantitated in the assays of this invention.

Representative methods and compositions according to this invention include those for identifying patients who have one or more malignant tumors. An exemplary method comprises the steps of detecting the level of the EGFr
15 ectodomain protein as described herein in a sample of a patient's body fluid and determining whether that level is elevated above normal. As shown herein, malignant tumors release a higher level of said EGFr ectodomain protein into body fluids than do benign tumors or normal tissues. Thus,
20 a higher than normal level of said EGFr ectodomain protein is indicative of the presence of one or more malignant tumors.

The invention provides for specific diagnostic/prognostic assays to detect and/or quantitate
25 said EGFr ectodomain protein in the body fluids of mammals, and thereby detect tumors, quantitate their growth, and provide valuable information for the diagnosis and prognosis of neoplastic disease. An elevated level of said EGFr ectodomain protein in a host's body fluid, that is, above
30 the normal background binding level, is indicative of neoplastic disease.

Preferably, the mammal being tested is human, and the body fluid assayed is serum or plasma. An important parameter of the status and survival probability of a
35 patient with neoplastic disease, such as breast, stomach including esophagus, colon, kidney, prostate, liver, urinary tract including bladder, lung, head and neck tumors and gynecologic cancers including ovarian, cervical, vaginal,

endometrial and vulval cancers, can be determined by testing a body fluid, preferably serum or plasma, from the patient for the presence of said EGFr ectodomain protein at elevated levels.

5 The invention further provides for methods and compositions to detect and/or quantitate said EGFr ectodomain protein in mammalian tissue extracts. Preferably, the extracts are from the tissues listed in the paragraph immediately above as representative of neoplastic
10 diseases that are preferred targets of the assays of this invention.

Exemplary means for detecting and/or quantitating said EGFr ectodomain protein whether in mammalian body fluids or in tissue extracts include affinity
15 chromatography, ligand binding assays, lectin binding assays and immunoassays, among other means. Immunoassays are a preferred means, most preferably a bridge immunoassay in a sandwich format. In such a bridge immunoassay, preferably an immunocomplex is formed in a liquid reaction between said
20 EGFr ectodomain protein and antibodies reactive therewith, wherein one of said antibodies is conjugated to a hapten and one of which is labeled, preferably enzymatically, and wherein a biotinylated bridge antibody raised against said hapten is then added to the reaction mixture and links said
25 immunocomplex to a solid phase that has been coated with avidin or streptavidin. In such immunoassays, antibodies reactive with epitope(s) on the EGFr ectodomain, preferably on said EGFr ectodomain protein, can be used. Further, in, for example, a competitive assay format, labeled proteins
30 and/or polypeptides from the EGFr ectodomain, preferably from the EGFr ectodomain protein, can be used to detect and/or quantitate said EGFr ectodomain protein in mammalian body fluids and/or tissue extracts. Also, alternatively, as a replacement for said labeled proteins and/or polypeptides
35 in such a representative competitive assay, labeled anti-idi type antibodies that have been prepared against antibodies reactive with epitope(s) on the EGFr ectodomain, preferably on said EGFr ectodomain protein, can be used.

Also provided are compositions comprising said EGFr ectodomain protein and/or fragments thereof in a substantially pure form. Those compositions can comprise said EGFr ectodomain protein, preferably glycosylated or partially glycosylated, and/or glycosylated and/or partially glycosylated fragments thereof, that have been isolated from one or more mammalian body fluids, preferably from serum, or from one or more mammalian tissue extracts. Said substantially pure EGFr ectodomain protein and/or fragments thereof, can be useful serologically in detecting and/or quantitating said EGFr ectodomain protein in mammalian body fluids.

Further, this invention provides for assays to detect and/or quantitate antibodies to said EGFr ectodomain protein in the body fluids of mammals. Such assay results especially in correlation with the results of assays of this invention that determine the level of said EGFr ectodomain protein in the body fluids of a patient provide important information for diagnosing and monitoring the patient's condition, deciding upon a course of treatment and in making a prognosis.

Still further, the assay results of this invention in indicating the presence and/or level of said EGFr ectodomain protein in the body fluids of humans and/or the presence and/or level of antibodies to said EGFr ectodomain protein are prognostically and diagnostically useful in conjunction with data from assays of other markers for neoplastic disease, such as, hormone receptors, for example, estrogen receptors (ER) and progesterone receptors (PR).

Exemplary of the diagnostic and prognostic indications that may be provided by the methods of this invention are the correlations of the results of assays according to this invention and the following representative associations referenced in the Background above: elevated levels of intact EGFr in tissues have been found to correlate with both a decreased chance of long term survival as well as a shortened time to relapse and poor responsiveness to endocrine therapy in breast and

endometrial cancers; also elevated levels of EGFr in tissues have been associated with good responsiveness of ovarian cancer to chemotherapy; also associated with such elevated levels of EGFr are squamous cell carcinomas of the cervix
5 and lung and invasiveness of bladder tumors.

The assays of this invention are useful for detection of malignant disease both pre- and post-operatively. For example, patients displaying elevated levels of said EGFr ectodomain protein, even at relatively
10 early stages of the disease, may, if the circumstances warrant, be treated more rigorously in order to increase their survival chances. Further, elevated levels of said EGFr ectodomain protein in a patient's body fluid, preferably serum or plasma, may indicate metastases
15 requiring immediate intervention, for example, systemic chemotherapy or radiation therapy. Further, by monitoring for either increases or decreases in levels of said EGFr ectodomain protein in a patient's body fluid, the physician may be able to determine if a patient is responding to
20 therapy or whether metastatic disease is present, stable or progressing. Still further, elevated levels of said EGFr ectodomain protein in a patient's body fluid may be used to detect and diagnose cancer including screening of populations for certain types of cancer.

25 The invention also provides for test kits that embody the assays of this invention wherein said test kits comprise antibodies reactive with said EGFr ectodomain protein. Further, said test kits can comprise labeled proteins and/or polypeptides from the EGFr ectodomain,
30 preferably from said EGFr ectodomain protein, and/or labeled anti-idiotypic antibodies prepared against antibodies that are reactive with said EGFr ectodomain protein. The assays can be solid phase assays but are not limited thereto, and can also be in a liquid phase format, and can be based on
35 enzyme linked immunosorbent assays (ELISAs), particle assays, radiometric or fluorometric assays either unamplified or amplified, using, for example, avidin/biotin technology.

DETAILED DESCRIPTION OF THE INVENTION

The concept underlying the many facets of this invention is the discovery that cancer patients have in their body fluids elevated levels of a portion of the EGFr which has a molecular weight in the range of from about 90 kilodaltons (kd) to about 115 kd, preferably from about 95 kd to about 105 kd, and more preferably about 100 kd. That portion of the EGFr comprises substantially the EGFr ectodomain. The inventors hereof show that one end of the amino acid sequence of said EGFr portion is substantially identical to the amino acid sequence reported for the N-terminus of the EGFr [Ullrich et al., Nature, 309: 418-425 (May 31, 1984)]. As indicated above, said portion of the EGFr which is found in mammalian body fluids is herein termed "the EGFr ectodomain protein".

"Substantially" as used in the context of "substantially comprises the EGFr ectodomain" indicates that although said EGFr ectodomain protein found in mammalian body fluids may not comprise the identical amino acid sequence or may comprise more or less amino acids than that reported for the EGFr ectodomain, it is recognizable as comprising a significant portion of the EGFr ectodomain with an N-terminus substantially identical to that reported for the N-terminus of the EGFr and with a molecular weight in the ranges stated above. It can be appreciated that a protein extant within the body fluids would be subject to degradative processes, for example, proteolytic, among other processes; further, it can be appreciated that a protein produced by neoplastic cell could be altered in sequence from that produced by a normal cell or by a cell in tissue culture. Thus, "the EGFr ectodomain protein" would encompass various species of the protein which may have different numbers of amino acids or different amino acids at some positions as well as different degrees of glycosylation from that reported for the EGFr ectodomain in the literature. Amino acid sequence variations in such a naturally occurring protein include, without limitation, amino acid substitutions, extensions, deletions,

truncations, interpolations and combinations thereof. Such variations fall within the contemplated scope of the invention provided the protein containing them has epitopes recognized by antibodies reactive with epitopes on EGFr ectodomain, preferably on the N-terminal 600 amino acids thereof. For example, the end of the EGFr ectodomain protein which is substantially identical in amino acid sequence to the N-terminus of the EGFr, may have a number of amino acids that differ from that reported in Ullrich et al., *id.*, or several amino acids could be missing from the N-terminus; nevertheless, said protein would still be "substantially" the EGFr ectodomain protein according to this invention. The stated molecular weight ranges encompass such various species of the EGFr ectodomain protein, wherein the predominant species has a molecular weight of about 100 kd.

The finding that said EGFr ectodomain protein is in human serum and other body fluids has opened the way for the development of novel methods and compositions for the diagnosis, prognosis and treatment of neoplastic disease in humans and other mammals. The assays of this invention are both diagnostic and/or prognostic, i.e., diagnostic/prognostic. The term "diagnostic/ prognostic" is herein defined to encompass the following processes either individually or cumulatively depending upon the clinical context: determining the nature of a disease, distinguishing one disease from another, forecasting as to the probable outcome of a disease state, determining the prospect as to recovery from a disease as indicated by the nature and symptoms of a case, monitoring the disease status of a patient, monitoring a patient for recurrence of disease, and/or determining the preferred therapeutic regimen for a patient. The diagnostic/prognostic methods of this invention are useful, for example, for screening populations for neoplastic disease, diagnosing neoplastic disease, monitoring the disease status of patients with neoplastic disease, and/or prognosticating the course of neoplastic disease.

Assays herein are provided not only to detect and/or quantitate in the body fluids of mammals, preferably humans, said EGFr ectodomain protein but also antibodies to said EGFr ectodomain protein. The assays of this invention
5 provide important information concerning the disease status of the patient, and are useful for screening mammals for neoplastic disease, monitoring the progress of the disease, and for prognosticating the course of the disease and deciding upon appropriate treatment protocols.

10 Correlation of the results from the assays to detect and/or quantitate said EGFr ectodomain protein and antibodies reactive therewith, provides a preferred profile of the disease condition of a patient. For example, a patient may present with a large tumor, but the patient's
15 level of said EGFr ectodomain protein may be relatively low. The lowness of the reading may be due to the patient's generation of antibodies to said EGFr ectodomain protein and not to the smallness of the tumor.

Further, correlation of the results of the assays
20 of the invention with those for other markers for neoplastic disease, such as assays measuring the levels of estrogen receptors (ERs) and progesterone receptors (PRs) in breast cancer, for example, as outlined above in the Background, can still further enhance the diagnostic/prognostic profile
25 of a cancer patient.

Assays for EGFr Ectodomain Protein in Mammalian Body Fluids

Non-invasive diagnostic/prognostic assays are provided to detect and/or quantitate said EGFr ectodomain
30 protein in the body fluids of mammals, preferably humans. It can be appreciated that said EGFr ectodomain protein can be broken down in a patient's body fluids into various fragments, and that said fragments may be detected and/or quantitated by the assays of this invention if they contain
35 one or more epitopes from the EGFr ectodomain protein that are recognized by the antibodies employed in this invention. For example, in a sandwich assay wherein two antibodies are used as a capture and a label antibody respectively, if both

epitopes recognized by those antibodies are present on a fragment of said EGFr ectodomain protein, said fragment would be detected and/or quantitated according to such as immunoassay. Such fragments which are detected and/or
5 quantitated according to methods of this invention are indicative of the presence of said EGFr ectodomain protein.

Such assays provide valuable means of monitoring the status of neoplastic diseases. In addition to improving prognostication, knowledge of the disease status allows the
10 attending physician to select the most appropriate therapy for the individual patient. For example, patients with a high likelihood of relapse can be treated rigorously, usually involving systemic chemotherapy and/or radiation therapy. When there is a lesser likelihood of relapse, less
15 aggressive therapies can be chosen. Because of the severe patient distress caused by the more aggressive therapy regimens, it would be desirable to distinguish with a high degree of certainty those patients requiring such aggressive therapies.

20 The present invention is useful for screening a wide variety of neoplastic diseases, including both solid tumors and hematopoietic cancers. Exemplary neoplastic diseases include carcinomas, such as adenocarcinomas and melanomas, and squamous cell carcinomas of the head and
25 neck; mesodermal tumors, such as neuroblastomas and retinoblastomas; sarcomas, such as osteosarcomas, Ewing's sarcoma, and various leukemias; and lymphomas. Of particular interest are gynecologic cancers including ovarian, cervical, vaginal, endometrial and vulval cancers;
30 gastrointestinal cancer, such as, stomach, colon and esophageal cancers; urinary tract cancer, such as, bladder and kidney cancers; skin cancer; liver cancer; prostate cancer; lung cancer; and breast cancer. Of still further particular interest are gynecologic cancers; breast cancer;
35 urinary tract cancers, especially bladder cancer; lung cancer; gastrointestinal cancer, such as, stomach, colon and esophageal cancers; and liver cancer. Even further of particular interest are gynecologic cancers and breast

cancer. Tumors which are known to overexpress intact EGFr may be good candidates for target neoplastic diseases for the assays of this invention, that is, such tumors may release into body fluids said EGFr ectodomain protein at elevated levels above normal. Particularly well studied are tumors of the breast and adenocarcinomas of the vulva which have been confirmed to overexpress EGFr. Preferably, in regard to tumors of the lung and cervix, squamous cell carcinomas have been particularly associated with elevated levels of EGFr. As outlined in the Background above, EGFr measurements have been determined to be markers for breast cancer and to provide valuable information diagnostically and prognostically, in correlation with other markers, such as, hormone receptor measurements, for example, with estrogen receptors (ERs) and progesterone receptors (PRs).

The body fluids that are of particular interest in assaying for said EGFr ectodomain protein according to the methods of this invention include blood, serum, semen, breast exudate, saliva, sputum, urine, cytosols, plasma, ascites, pleural effusions, amniotic fluid, bladder washes, bronchioalveolar lavages, and cerebrospinal fluid. Blood, serum and plasma are preferred, and serum is the most preferred body fluid for screening according to the methods of this invention. The assays of this invention may also be useful in detecting and/or quantitating said EGFr ectodomain protein in tissue extracts.

From a knowledge of the structure of the external domain of the EGFr, a number of monoclonal or polyclonal antibodies can be generated that specifically recognize that protein. Because said EGFr ectodomain protein is found to exist freely in the biological fluids of mammals, it is possible to detect and/or quantitate the levels of that protein. Utilizing current antibody detection techniques that can quantitate the binding of monoclonal antibodies, made specifically to epitopes on the external domain of the EGFr, preferably to epitopes on the N-terminal 600 amino acids, one can determine the amount of said EGFr ectodomain protein in the fluids of cancer patients.

Such an assay can be used to detect tumors, quantitate their growth, and help in the diagnosis and prognosis of the human disease. The assays can also be used to detect the presence of cancer metastasis, as well as
5 confirm the absence or removal of all tumor tissue following surgery, cancer chemotherapy and/or radiation therapy. It can further be used to monitor cancer chemotherapy and tumor reappearance. Representative immunoassays involve the use of monoclonal and/or polyclonal antibodies which can be
10 appropriately labeled to detect and/or quantitate said EGFr ectodomain protein in the body fluids of mammals.

One preferred method involves, as a first step, obtaining a purified amount of the external domain of the EGFr (procedure detailed below) and using it as an immunogen
15 to generate monoclonal antibodies in mice or other suitable hosts. Alternatively, in another preferred method, whole cells expressing intact EGFr on their membrane surface can be used as a source of antigen. Still further, in another preferred method, purified EGFr (for example, solubilized
20 from membranes in an A431 lysate preparation and then purified as detailed below) is used as the immunogen. It is possible that numerous monoclonal antibodies could be generated to recognize different epitopes on said EGFr ectodomain protein, preferably on the N-terminal 600 amino
25 acids, and such monoclonal antibodies can be used either singularly or in combination as a cocktail to increase the specificity and sensitivity of an assay.

It is preferred in a format where more than one antibody is used as, for example, in a sandwich format, in a
30 competitive/sandwich format, or where a cocktail of antibodies are used, that the epitopes with which the antibodies are reactive be widely spaced. One representative and preferred assay of this invention comprises the use of three antibodies to epitopes that are
35 sufficiently spatially removed from each other to produce effective results.

Besides using the intact EGFr, purified EGFr and/or whole external domain as an immunogen, fragments of

the EGFr ectodomain or from the EGFr ectodomain protein, or protein/polypeptide generated by recombinant DNA means or otherwise biologically from the EGFr ectodomain, can be also used to generate specific monoclonal and/or polyclonal
5 antibodies. Also, polypeptides corresponding to various sequences within the external domain sequence, preferably within the N-terminal 600 amino acids, small polypeptides (peptides) attached to an appropriate carrier protein, could be used as a source of immunogens. In all cases, the
10 antibodies generated that are selected would have a specificity such that they have very limited cross-reactivity with other proteins present on the surface of both tumors and non-tumor cells. They would preferably not, for example, react with proteins which are present on
15 the surface of many normal cells.

The diagnostic/prognostic assay itself would typically involve obtaining a small amount of body fluid, preferably serum, from the preferably human host. The presence of said EGFr ectodomain protein in the serum can
20 then be detected and/or quantitated using a number of well-defined antibody diagnostic assays. Those can be Western blots, ELISAs (enzyme-linked immunosorbent assays), RIA assays (radioimmunoassay), competitive EIA or dual antibody sandwich assays, all commonly used in the
25 diagnostic industry. In such immunoassays, the interpretation of the results is based on the assumption that the antibody or antibody combination will not cross-react with other proteins and protein fragments present in the serum that are unrelated to EGFr.

Below under the heading Bridge Immunoassay and in
30 Example 2, details of a preferred format for the assays of this invention -- a sandwich bridge immunoassay -- are provided. Many other formats for detection and/or quantitation of said EGFr ectodomain protein according to
35 this invention in body fluids are of course available, including, for example conventional sandwich, competitive, and competitive/sandwich formats among others as indicated above.

Representative of one type of ELISA test is a format wherein a microtiter plate is coated with antibodies to EGFr extracellular domain, to purified EGFr and/or antibodies to whole cells expressing, preferably overexpressing EGFr, (that is, to intact EGFr wherein "intact" in this context indicates that the extracellular domain is expressed on the surface of cells), and to said microtiter plate is added a sample of a patient's serum. After a period of incubation permitting any antigen to bind to the antibodies, the plate is washed and another set of anti-EGFr extracellular domain antibodies and/or other antibodies that recognize epitopes on the EGFr ectodomain protein, which antibodies are linked to an enzyme, is added, incubated to allow a reaction to take place, and the plate is then rewashed. Thereafter, enzyme substrate is added to the microtiter plate and incubated for a period of time to allow the enzyme to work on the substrate, and the absorbance of the final preparation is measured. A large change in absorbance indicates a positive result.

It is also apparent to one skilled in the art of diagnostic /prognostic assays that antibodies to EGFr extracellular domain proteins and/or polypeptides can be used to detect and/or quantitate the presence of said EGFr ectodomain protein in the body fluids of patients. In one such embodiment, a competition immunoassay is used, wherein an EGFr extracellular domain protein/polypeptide is labeled, and a body fluid is added to compete the binding of the labeled EGFr extracellular domain protein/polypeptide to antibodies specific to the EGFr extracellular domain. Such an assay could be used to detect and/or quantitate said EGFr ectodomain protein.

Once antibodies having suitable specificity have been prepared, a wide variety of immunological assay methods are available for determining the formation of specific antibody-antigen complexes. Numerous competitive and non-competitive protein binding assays have been described in the scientific and patent literature, and a large number of such assays are commercially available. Exemplary

immunoassays which are suitable for detecting a serum antigen include those described in U.S. Patent Nos. 3,791,932; 3,817,837; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 5 3,984,533; 3,996,345; 4,034,074; and 4,098,876.

Antibodies employed in assays may be labeled or unlabeled. Unlabeled antibodies may be employed in agglutination; labeled antibodies may be employed in a wide variety of assays, employing a wide variety of labels.

10 Suitable detection means include the use of labels such as radionuclides, enzymes, fluorescers, chemiluminescers, enzyme substrates or co-factors, enzyme inhibitors, particles, dyes and the like. Such labeled reagents may be used in a variety of well known assays, such 15 as radioimmunoassays, enzyme immunoassays, e.g., ELISA, fluorescent immunoassays, and the like. See for example, U.S. Patent Nos. 3,766,162; 3,791,932; 3,817,837; and 4,233,402.

Another means of detecting and/or quantitating 20 said EGFr ectodomain protein may be the use of EGF affinity chromatography, for example as described in Buhrow et al., J. Biol. Chem., 257: 4019-4022 (1982) as a ligand binding assay. A further means of detecting and/or quantitating said EGFr ectodomain protein be the use of lectin binding 25 assays.

Bridge Immunoassay

A preferred assay for said EGFr ectodomain protein according to this invention is the bridge immunoassay 30 format. The bridge immunoassay provides an immunoassay methodology wherein a universal capture system is employed; an immunocomplex containing the sample analyte, the EGFr ectodomain protein, is formed in solution and then captured and insolubilized by a universal bridge receptor which links 35 the immunocomplex to a solid phase through a first receptor thereto.

The bridge immunoassay provides a capture system comprising at least three receptors. The first receptor is

bound to a solid phase and has as its ligand a second receptor or a ligand conjugated to said second receptor, wherein said second receptor is a bridge receptor, preferably an anti-hapten bridge receptor. The bridge
5 receptor binds to a ligand conjugated to a sample analyte receptor, the third receptor. The third receptor has as its ligand either the analyte under assay or a further receptor which has as its ligand the analyte under assay.

The bridge immunoassay can be used in any standard
10 immunoassay format including sandwich, competitive and sandwich/competitive immunoassay formats. In a sandwich immunoassay format, a labeled fourth receptor, in this case to the sample analyte, is used to form a sandwich
immunocomplex in solution with the analyte and the third
15 receptor. After the initial liquid phase reaction when the immunocomplex sandwich is formed, the bridge receptor (second receptor) is added to the liquid sample where it binds to a ligand conjugated to the sample analyte receptor (third receptor) and pulls the immunocomplex out of solution
20 by binding to the first receptor, which is bound to the solid phase. The amount of label (on the fourth receptor) that is immobilized upon the solid phase or remaining in the liquid phase can then be measured by conventional means to detect whether or not the analyte (said EGFr ectodomain
25 protein in this invention) had been present in the sample and if so, determine the amount of analyte that had been present.

There are many different embodiments for the bridge immunoassay. A preferred embodiment to assay for the
30 EGFr ectodomain protein of this invention is the bridge immunoassay in a sandwich format. In such a preferred bridge immunoassay format, the first receptor is preferably avidin or streptavidin, more preferably streptavidin [Zymed Laboratories, South S.F., CA (USA)] coated upon a solid
35 phase, more preferably coated to the inner walls of polystyrene test tubes [most preferably, 12 mm x 75 mm; Nunc star tubes; Nunc, Napierville, IL (USA); catalog #470319] wherein the reaction volume is 0.5 mL and wherein 2.5 µg per

tube of streptavidin was offered. In such a preferred assay, the second receptor (bridge receptor) is preferably a biotinylated bridge antibody, preferably a monoclonal antibody, which was raised to a hapten conjugated to an appropriate carrier protein, preferably fluorescein isothiocyanate (FITC) conjugated to KLH, preferably purified by HPLC. Said bridge receptor is at a concentration preferably of about 800 ng per tube. In such a preferred assay, the third receptor is an antibody, preferably a monoclonal antibody that recognizes an epitope on the ectodomain of the EGFr, preferably on the N-terminal 600 amino acids of the ectodomain, more preferably the monoclonal antibody, 31G7 (specified below), to which FITC is conjugated. The amount of said third receptor is preferably proportional to the amount of the bridge receptor (second receptor). It is preferred that there be about one to about 20 times more bridge antibody than third receptor (capture antibody), and still more preferred that there be about 4 to about 12 times more bridge antibody than third receptor on a molar basis. In such a preferred assay, the fourth receptor is an enzyme-labeled monoclonal antibody which recognizes an epitope on the ectodomain of the EGFr, preferably on the N-terminal 600 amino acids of the ectodomain, more preferably monoclonal antibody 4C7 (specified below); the enzymatic label on said fourth receptor is preferably horseradish peroxidase.

A preferred method of preparing the enzyme-labelled antibody for such an assay is the use of a heterobifunctional methodology of conjugation [Sullivan and Marks, Methods in Enzymology: (9) Methods for the Preparation of Enzyme-Antibody Conjugates for Use in Enzyme Immunoassay, 73: 147 and ff. (1981)]. More specifically, it is preferred that the enzyme, preferably HRP, be thiolated using N-succinimidyl S-acetylthioacetate (SATA) as described in Duncan et al., Anal. Biochem., 132 : 68-73 (1983), and that it be coupled with an antibody activated by using one of the following three or ss-linking reagents each of which is described in the article in brackets thereafter:

succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) [Tsurata et al., J. Histochem Cytochem., 33 (8) : 767 and ff. (1985)]; m - maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) [Liu et al., Biochem., 18 (4) : 690 and ff. (1979)]; and N-succinimidyl bromoacetate (NSBA) [Bernatowicz and Matsueda, Anal. Biochem., 155 : 95-102 (1986)].

Example 2 below provides the details of a preferred protocol for a bridge immunoassay to detect and/or quantitate the EGFr ectodomain protein in the body fluids of mammals according to this invention.

Assays for Antibodies to the EGFr Ectodomain Protein

As indicated above, the level of antibodies to said EGFr ectodomain protein in a patient's body fluids is an important parameter in screening for neoplastic disease, monitoring, and prognosticating the course of the disease and on deciding upon a course of treatment. Such antibodies can be detected and/or quantitated by conventional means known in the art that are analogous to those means outlined above for detecting and quantitating said EGFr ectodomain protein according to this invention. A representative assay to detect and/or quantitate such antibodies is a competition assay in which a labeled EGFr extracellular domain protein/polypeptide, preferably from the N-terminal 600 amino acids, is precipitated by antibodies in patient serum in competition with a known quantity of monoclonal antibodies recognizing epitope(s) on the EGFr extracellular domain, preferably on the N-terminal 600 amino acids. One skilled in the art could adapt any of the formats outlined and referred to in the above section to detect and/or quantitate antibodies to said EGFr ectodomain protein. Further, the results of such assays for antibodies to the EGFr ectodomain protein can also be correlated with other indicators of neoplastic disease. For example, in the case of breast cancer, the results of the assays for said antibodies may be correlated with results of assays for hormone receptors, such as ERs and PRs.

Test Kits

The above outlined assays can be embodied in test kits to detect and/or quantitate said EGFr ectodomain protein in mammalian, preferably human, body fluids wherein
5 such test kits can comprise antibodies, polyclonal and/or monoclonal, that recognize epitopes on said EGFr ectodomain protein, made against, for example, purified EGFr, against purified EGFr extracellular domain, against EGFr
10 extracellular domain proteins and/or polypeptides, and/or against whole cells expressing EGFr (that is, to intact EGFr). Such diagnostic/prognostic test kits can further comprise one or more other sets of antibodies, polyclonal and/or monoclonal, for a sandwich format wherein said other antibodies similarly recognize such epitopes on said EGFr
15 ectodomain protein, and are appropriately labeled.

Test kits for an assay format wherein there is competition between a labeled EGFr extracellular domain protein/polypeptide and said EGFr ectodomain protein in the sample, for binding to an antibody, can comprise the
20 combination of the labeled protein/polypeptide and the antibody in amounts which provide for optimum sensitivity and accuracy. Said antibody which recognizes appropriate epitopes can be either polyclonal and/or monoclonal.

Further, said test kits can comprise proteins
25 and/or polypeptides from said EGFr ectodomain protein, alone or in combination with the aforementioned antibodies. Anti-idiotypic antibodies to the anti-EGFr ectodomain antibodies, can be substituted for EGFr extracellular domain proteins and/or polypeptides in such test kits.

30

Abbreviations

The following abbreviations are used herein:

	AEV	avian erythroblastosis virus
	ATCC	American Tissue Culture Collection
35	BSA	bovine serum albumin
	°C	degrees centigrade
	cm	centimeter
	DAB	diaminobenzidine t trahydrochloride

	DNS	1,4-N,N- dimethyl amino-napthalene sulfonic acid
	EDTA	ethylenediaminetetraacetic acid
	EGF	epidermal growth factor
5	EGFr	epidermal growth factor receptor
	EIA	enzyme immunoassay
	ELISA	enzyme labeled immunosorbent assay
	ER	estrogen receptor
	ERRP	epidermal growth factor receptor-related protein
10	FITC	fluorescein isothiocyanate
	fmol	femtomole
	HEPES	4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid
15	HPLC	high pressure liquid chromatography
	hr	hour
	HRP	horseradish peroxidase
	IRMA	immunoradiometric assay
	kd	kilodalton
20	KLH	keyhole limpet hemacyanin
	L	liter
	M	molar
	mA	milliampere
	MAb	monoclonal antibody
25	MBS	<u>m</u> -maleimidobenzoyl-N-hydroxysuccinimide ester
	mg	milligram
	min	minute
	mL	milliliter
	mM	millimolar
30	mm	millimeter
	NFDM	non-fat dried milk
	ng	nanogram
	NHS	normal human serum
	nm	nanometer
35	NP-40	Nonidet P-40 (non-ionic detergent from Sigma Chemical Co. in St. Louis, MO)
	NSBA	N-succinimidyl bromoacetat

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	PBS	phosphate-buffered saline
	PEG	polyethylene glycol
	PMSF	phenylmethanesulfonylfluoride
	PR	progesterone receptor
5	RIA	radioimmunoassay
	rpm	revolutions per minute
	RPMI	Roswell Park Memorial Institute 1640 media
	SATA	N-succinimidyl S-acetylthioacetate
	S.D.	standard deviation
10	SDS	sodium dodecyl sulfate
	SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
	sec	second
	SMC	succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate
15	TGF- α	transforming growth factor-alpha
	TMB	3,3',5,5'- tetramethyl benzidine
	TRIS	tris (hydroxymethyl) aminomethane or amino-2-hydroxymethyl-1,3-propanediol
20	TWEEN-20	polyoxyethylene-sorbitan monolaurate (non-ionic detergent from Sigma Chem. Co., St. Louis, MO)
	μ g	microgram
	μ L	microliter
25	V	volt
	vol	volume
	xg	times gravity

Specifically, the following one-letter codes are used to represent amino acids:

30	A = alanine	M = methionine
	C = cysteine	N = asparagine
	D = aspartic acid	P = proline
	E = glutamic acid	Q = glutamine
	F = phenylalanine	R = arginine
	G = glycine	S = serine
35	H = histidine	T = threonine
	I = isoleucine	V = valine
	K = lysine	Y = tyrosine

L = leucine

W = tryptophan

Cell Lines

The following cell lines were used in the
5 experiments herein described:

- | | | |
|---------------|---|---|
| A431 | - | Human epidermoid carcinoma cell line
obtained from the ATCC, catalog #CRL
1555 |
| | | |
| 10 SW620 | - | Human colon adenocarcinoma cell line
derived from a lymph node metastasis,
obtained from the ATCC, catalog #CCL 227 |
| | | |
| 15 SP2/0-Ag14 | - | Non-secreting mouse myeloma cell line,
obtained from the ATCC, catalog #CRL
1581 |

Preparations and Antibodies

The following preparations and antibodies were
20 used in the examples below.

Preparation of A431 and SW620 Cell Lysates

A431 cell lysates were prepared from confluent
cell monolayers grown in 54 T150 tissue culture flasks. The
25 cells were rinsed twice with cold PBS with 1 mM PMSF
(phenylmethylsulfonyl fluoride) (Sigma, St. Louis, MO) using
approximately 10 mLs per wash. Five mLs of the wash
solution were added to each flask, and the cells were
removed with a cell scraper. The cells were pelleted and
30 then resuspended into 15 mLs homogenization buffer (10 mM
Tris-HCl, pH 7.4, 1.5 mM EDTA, and 1 mM PMSF) and disrupted
with a Polyron homogenizer (Brinkman Instruments,
Switzerland) using five 10-sec pulses at setting 4. The
homogenate was pelleted in a Sorvall RC5C centrifuge [Du
35 Pont Company, Wilmington, DE (USA)] at 14,000 rpm for 15
min. The pellet was resuspended in 10 mL extraction buffer
(50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% TRITON
X-100) [Sigma, St. Louis, MO (USA)], and 1 mM PMSF (Sigma

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Chem. Co.), mixed well and set on ice for 15 min. The extract was centrifuged in a Beckman microfuge 12 [Beckman Instruments, San Francisco, CA (USA)] for 15 min at high speed. The supernatant (lysate) was aliquoted and stored at -70°C.

- 5 Cell lysates from confluent monolayers of SW620 cells were also prepared as described for the A431 cells.

Preparation of Polyclonal Antibodies

- 10 The techniques for making polyclonal antibodies are conventional in the immunoassay art. Anti-peptide polyclonal antibodies are also made by conventional methods in the art as described in European Patent Publication No. 44,710 (published Jan. 27, 1982). Briefly, such anti-peptide antibodies are prepared by selecting a peptide
15 from the EGFr amino acid sequence (Ullrich et al., supra), chemically synthesizing it, conjugating it to an appropriate immunogenic protein and injecting it into an appropriate animal, usually a rabbit or a mouse; then, either polyclonal or monoclonal antibodies are made, the latter by the
20 Kohler-Milstein procedure.

- Chemical synthesis of a peptide is conventional in the art and can be accomplished, for example, by the Merrifield solid phase synthesis technique [Merrifield, J. Am. Chem. Soc., 85:2149-2154 (1963); Kent et al., Synthetic Peptides in Biology and Medicine, 29f.f., eds. Alitalo et al., (Elsevier Science Publishers 1985); and Haug, ABL, 40-47 (Jan/Feb. 1987)].
25

- Techniques of chemical peptide synthesis include using automatic peptide synthesizers employing commercially
30 available protected amino acids; such synthesizers include, for example, Biosearch [San Rafael, CA (USA)] Models 9500 and 9600; Applied Biosystems Inc. [Foster City, CA (USA)] Model 430; MilliGen [a division of Millipore Corp.; Bedford, MA (USA)] Model 9050; and Du Pont's Ramp (Rapid Automated
35 Multiple Peptide Synthesis) [DuPont Company, Wilmington, DE (USA)].

All the polyclonal antibodies used for this invention were screened against purified EGFr, A431 cell lysates and SW620 lysates (from human colon adenocarcinoma).

5 Preparation of Polyclonal Antibody EGFr #2

New Zealand white rabbits were immunized with 5 μ g of purified EGFr derived from A431 cells as described below. The initial immunization consisted of the antigen emulsified 1:1 (vol/vol) in Freund's complete antigen and injected into
10 the popliteal lymph nodes. Subsequent boosts were given intraperitoneally at two week intervals, with the antigen emulsified in incomplete Freund's adjuvant. The animals were bled every two weeks by ear vein, and the sera assayed by ELISA against purified EGFr, against A431 cell lysates
15 and against SW620 cell lysates. The procedure for those ELISAs is described within the preparation of the monoclonal antibodies below.

Preparation of Polyclonal Antibody PEP-EGFr #1

20 PEP-EGFr#1 polyclonal antiserum was made by immunizing New Zealand white rabbits with the peptide TBI 487 (a 20-mer at the C-terminal end of the EGFr cytoplasmic domain, amino acids 1167-1186, TAENAEYLRVAPQSSEFIGA) after conjugation to KLH with glutaraldehyde. The peptide was
25 provided by Protein Chemistry, Triton Biosciences [Alameda, CA (USA)]. An immunization scheme similar to the one described above was used. For the initial immunization, 250 μ g of peptide equivalent was used; subsequent boosts used 100 μ g. The animals were bled, and the sera assayed as
30 indicated above for polyclonal antibody EGFr #2.

Preparation of Polyclonal Antibody EGFr 421 #1

EGFr 421 #1 polyclonal antiserum was made by immunizing New Zealand white rabbits with the peptide TBI
35 421 (a 17-mer in the C-terminal region of the EGFr cytoplasmic domain, amino acids 984-1000, DVVDADEYLIPQQGFFS) after conjugation to KLH with glutaraldehyde. The peptide was provided by Protein Chemistry, Triton Biosciences

[Alameda, CA (USA)]. The amounts of antigen used, and the immunization schedule were identical to that used to make PEP-EGFr #1. The animals were bled, and the sera assayed as indicated above for polyclonal antibody EGFr #2.

5

Preparation of Monoclonal Antibodies

Monoclonal antibodies useful in the present invention are obtained by well known processes as described in, for example, Gutfre and Milstein, "Preparation of
10 Monoclonal Antibodies: Strategies and Procedures, " in Methods in Enzymology: Immunochemical Techniques, 73:1-46 (Langone and Vanatis eds. Academic Press 1981); and in the classic reference, Milstein and Kohler, Nature, 256:495-497 (1975).

15

All the monoclonal antibodies used for this invention were screened against purified EGFr, A431 cell lysates and SW620 lysates.

MAbs 4C7, 26C3, 29C12 and 31G7

20

The monoclonal antibody 4C7 was made by fusing splenocytes from Balb/c mice immunized with A431 cells to the myeloma cell line SP2/0-Ag14 (ATCC CRL 1581 non-secreting mouse myeloma). Monoclonal antibodies 26C3, 29C12, and 31G7 were made by fusing to said myeloma cell
25 line splenocytes from Balb/c mice immunized with EGFr purified from A431 cells.

30

Balb/c mice were immunized intraperitoneally with either 10^7 A431 cells or 5 μ g purified EGFr emulsified 1:1 (vol/vol) in RIBI adjuvant [RIBI ImmunoChem Research, Inc.,
Hamilton, MT (USA)]. The animals were boosted every two weeks with emulsified antigen. Sera were collected from the tail vein every two weeks and tested for reactivity in an ELISA assay (described below) against lysates of A431 and SW620 cells (ATCC CCL 227, human colon adenocarcinoma
35 derived from a lymph node metastasis), as well as against purified EGFr. Animals with positive titers were boosted intravenously with antigen in PBS and sacrificed 4 days later for fusion. Splenocytes were fused with the SP2/0-Ag

14 cells at a ratio of 4:1 with a 50% polyethylene glycol 4000 solution [EM Sciences, Cherry Hill, NJ (USA)] using the procedure of Kohler and Milstein [Nature, 256:495-497 (1975)]. Fused cells were gently washed and plated into
5 96-well plates at 2.5×10^5 splenocytes per well in RPMI. On days 1, 2, 3, 4, and 6 following fusion, half the media was removed and replaced with media supplemented with 0.1 mM hypoxanthine and 5.8 uM azaserine (Sigma, St. Louis, MO).

Supernatants were tested for reactivity in the
10 ELISA assay on day 11. Vinyl assay plates (96-well) [Costar; Cambridge, MA (USA)] were coated with A431 lysate (250 ng/well), SW620 lysate (250 ng/well), or purified EGFr (25 ng/well) overnight. After blocking with 4% non-fat dry milk, 0.05% TWEEN-20 in PBS for 1 hr at room temperature, 10
15 μ L of supernatant was added to 50 μ L PBS and allowed to incubate for 1 hr at 37°C. After washing in PBS with 0.05% TWEEN-20, the plates were incubated with 50 μ L of rabbit anti-mouse IgG-horse radish peroxidase conjugate [Jackson ImmunoResearch, West Grove, PA (USA)] at a 1:3000 dilution
20 in PBS for 30 min at 37°C for 1 hr. Following the final wash, 50 μ L of the TMB (3, 3', 5, 5' tetramethylbenzidine) substrate was added to the wells for a 15 min incubation. The color development was quenched by the addition of 50 μ L 1 M phosphoric acid. The plates were read on a Molecular
25 Devices Vmax kinetic microplate reader [Menlo Park, CA (USA)] at an optical density of 450 nm. Hybridomas were chosen that reacted positively on the A431 lysate and EGFr coated plates, yet negatively on the SW620 coated plates. All positive cell lines went through two cloning rounds by
30 limiting dilution to ensure monoclonality.

Screening of Antibodies for Recognition of EGFr Ectodomain

EGFr ectodomain was purified from A431 tissue culture supernatant as described below. The antibodies were
35 screened in an ELISA assay against the purified EGFr ectodomain as described below. Of the monoclonal antibodies to EGFr all but 29C12 recognized epitopes on the EGFr ectodomain.

RB Monoclonal 20B3.

The RB monoclonal 20B3 to which Example 1 below refers is a mouse monoclonal made to a peptide encoded by a retinoblastoma gene. The RB monoclonal is available from Triton Diagnostics Inc. [Alameda, CA (USA)].

MAb 225 [commercially available from Oncogene Sciences in Manhasset, NY (USA)] recognizes an epitope in the binding site of the EGFr ectodomain. Its characterization is described in Masui et al., Cancer Res., 44: 1002-1007 (1984).

EGFr Ectodomain Purification

As indicated above in the Background, Weber and Gill [Science, 224: 294-297 (April 20, 1984)] identified in the supernatant of A431 cells, a soluble 105-kilodalton protein which by the criteria of EGF binding, recognition by monoclonal and polyclonal antibodies to the EGFr, amino-terminal sequence analysis and carbohydrate content, is related to the cell surface domain of EGFr. Weber and Gill called that protein an "extracellular EGF receptor-related protein (ERRP)". A similar procedure herein was used to purify a protein of similar molecular weight (100-105 kd band) from the supernatant of A431 cells wherein said protein reacted with EGFr #2 (polyclonal antiserum made to EGFr purified from A431 cell membranes) in a Western blot, but was not recognized by the two polyclonal antisera (PEP-EGFr #1 and EGFr 421 #1) made to peptides respectively from the C-terminal end of EGFr and within the EGFr cytoplasmic domain.

The N-terminal 20 residues of that protein purified from the A431 supernatant was sequenced on automatic sequencing equipment (Applied Biosystems, Foster City, CA), and those residues were found to be identical to N-terminal residues described by Ullrich et al., supra for EGFr ectodomain. Thus, that protein purified from the supernatant of A431 cells is herein termed to be "purified EGFr ectodomain".

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The procedure used to purify the EGFr ectodomain is as follows: A431 cells (ATCC CRL 1555, human epidermoid carcinoma cells derived from an 85-year-old human female) were grown to confluency, and supernatants were harvested from the tissue culture flasks and filtered through a 0.22 micron cellulose acetate filter to remove cell debris. The media was then concentrated ten-fold on a Minitan system [Millipore Corp., Bedford, MA (USA)]. The media was applied to a 1.0 x 5 cm column packed with 5 mL MAb 225-Sepharose affinity gel (wherein the Sepharose is available from Sigma Chem. Co. and is linked to MAb 225 according to manufacturer's instructions). The column was loaded at a flow-rate of approximately 2 mL/min and then thoroughly washed with HTNG (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% TRITON, 10% glycerol) buffer until a stable baseline absorbance at 280 nm as monitored on a Frac-100 fraction collector [Pharmacia, Piscataway, NJ (USA)] was attained. Nonspecific binding was removed by washing the column with 1 M NaCl, followed by washing with HTNG until the baseline was attained. The EGFr ectodomain was eluted from the column with 10 column volumes of 50 mM triethylamine, pH 10.5. It was then washed with 20 column volumes of PBS containing 0.05% NaN₃ and stored. Fractions were monitored for the presence of the purified EGFr ectodomain by dotblot with ¹²⁵I TGF- α . Those containing the purified EGFr ectodomain were dialyzed against HTNG buffer and concentrated in a Centriprep 30 [Amicon, Beverly, MA (USA)].

EGFr Purification Method

EGFr was solubilized from the membranes in an A431 lysate preparation by incubation in extraction buffer (10 mM HEPES, pH 7.5, 4 mM benzamidine, 3mM EDTA, 0.5 units/mL aprotinin, 1% TRITON X-100, 10% glycerol, 100 mM NaCl, and 5 μ g/mL leupeptin) [all chemicals available from Sigma, St. Louis, MO] for 15 min on ice. After centrifugation at 10,000 x g for 10 min, the supernatant was collected, applied to a MAb 225-Sepharose column, and allowed to recirculate slowly overnight. The purification procedure of

EGFr using immunoaffinity chromatography was identical, from this step on, to that described for the EGFr extracellular domain (above). Downward et al., *Nature*, 307: 521-527 (1984) also describes a method for purifying EGFr.

5

ELISA Assay for Purified EGFr Ectodomain

Vinyl assay plates (96-well) [Costar, Cambridge, MA (USA)] were coated with 5 ng/well purified EGFr ectodomain diluted in PBS and incubated overnight at room temperature. The plates were blocked with 4% non-fat dry milk, 0.05% TWEEN-20 in PBS for 1 hr at room temperature followed by a wash in PBS, 0.5% TWEEN-20. Fifty μ L of hybridoma supernatant were added to each well and allowed to incubate at 37°C for 1 hr. After washing, the plates were incubated with 50 μ L of rabbit anti-mouse IgG-horse radish peroxidase conjugate [Jackson ImmunoResearch, West Grove, PA (USA)] at a 1:3000 dilution in PBS for 30 min at 37°C. Following the final wash, the TMB (3,3',5,5' tetramethylbenzidine) substrate [Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD (USA)] was added at 50 μ g/well for 15 min. The color development was quenched by the addition of 50 μ L of 1 M phosphoric acid. Plates were read on a Vmax kinetic plate reader [Molecular Devices, Menlo Park, PA (USA)] at an optical density of 450 nm.

25

Tube Coating with Streptavidin

To Nunc star tubes (12 mm x 75 mm; Nunc catalog #470319) is added one-half mL of streptavidin in a coating buffer of sodium bicarbonate at pH 9.6 at a concentration of 5 μ g of streptavidin per mL. The tubes are covered and stored at 2°C to 8°C for about 16 to 24 hours. The liquid is then aspirated from the tubes which are then washed twice with 2 mL of phosphate buffered saline and 0.05% TWEEN 20 wash buffer.

35

Two mL of PBS- 1% bovine serum albumin (BSA) blocking solution are added to the tubes which are then incubated at room temperature for about two to about four hours. The liquid is then aspirated from the tubes which

are then washed twice with a 1% glucose solution. The liquid is then aspirated. The tubes are then uncovered and placed in a drying chamber until thoroughly dry, which occurs overnight. The dried tubes are then placed in
5 heat-sealable Mylar (moisture-resistant plastic) pouch with a dessicant pack and maintained therein until ready for use in an assay according to this invention.

EGFr Ectodomain Protein/Polypeptide Production

10 As indicated above the complete amino acid sequence for the EGFr is known [Ullrich et al., Nature, supra]. EGFr extracellular proteins/polypeptides can be prepared recombinantly, synthetically or otherwise biologically. Exemplary of biologic means other than
15 recombinant means to prepare a desired polypeptide or protein is to subject to selective proteolysis a longer EGFr extracellular polypeptide or protein containing the desired amino acid sequence. For example, said longer polypeptide or protein can be split with chemical reagents or with
20 enzymes.

Synthetic formation of a polypeptide or protein requires chemically synthesizing the desired chain of amino acids by methods well known in the art, and discussed above under Preparation of Polyclonal Antibodies.

25 A "polypeptide" is defined herein to be a chain of amino acids covalently bound by peptide linkages composed of 50 or less amino acids. A "protein" is herein defined to be a chain of amino acids covalently bound by peptide linkages composed of more than 50 amino acids.

30 It is understood that, in some instances, amino acid sequence variations of naturally occurring proteins and polypeptides may be antigenic. Possible sequence variations include, without limitation, amino acid substitutions, extensions, deletions, truncations, interpolations and
35 combinations thereof. Such variations are acceptable according to this invention provided the protein or polypeptide containing them is immunogenic and antibodies

elicited by such a polypeptide or protein recognize epitopes on the naturally occurring EGFr ectodomain protein.

Further, it will be appreciated that the amino acid sequence of the EGFr extracellular domain can be modified by genetic techniques. One or more amino acids can be deleted or substituted. Such amino acid changes, especially if in a region which is not within an epitope of the polypeptide, may not cause any measurable change in the serological and/or immunogenic activity of a protein or polypeptide. The resulting protein or polypeptide will have substantially the same amino acid sequence and substantially the same biological activity.

The following examples are presented to help in the better understanding of the subject invention and for purposes of illustration only. They are not to be construed as limiting the invention in any manner.

EXAMPLE 1

Human Serum assayed in EGFr ELISA

Nunc certified polystyrene microtiter plates (Nunc Inter Med; Kamstrup, Denmark) were coated overnight at 4°C with two EGFr monoclonals (29C12 and 4C7) and one negative control monoclonal (RB monoclonal 20B3) at 1 µg/well (100 µL) in PBS (10 mM sodium phosphate, pH 7.4, 0.1 M NaCl). The coating solution was decanted, and the plates were blocked for 1.5 hours at room temperature with 200 µL 4% non-fat dried milk (NFDM) in PBS. The plates were decanted and then washed five times with wash buffer (PBS with 0.05% TWEEN-20, a non-ionic detergent from Sigma Chem. Co., St. Louis, Mo.).

A431 lysate was diluted in assay buffer (0.5% NFDM in PBS with 0.05% TWEEN-20) to yield standards ranging from 10 to 0.1 µg/mL of total protein. Normal human serum [NHS from Bioheme, Salt Lake City, UT (USA)] was diluted 1:100 in assay buffer. The A431 lysate and NHS were added to the plates in duplicate (100 µL/well) and incubated for 1 hour at 37°C. The plates were then wash d 5 times with the wash buffer.

Biotinylated monoclonal antibodies to EGFr--4C7, 26C3 and 31G7 -- were added to respective wells and incubated for 1 hour at 37°C. The plates were again washed 5 times and a goat anti-biotin horseradish peroxidase (HRP) conjugate [Zymed Laboratories, South San Francisco, CA (USA)] was added and incubated for 30 minutes at 37°C. The plates were washed and KPL [Kirkegaard and Perry Laboratories, MD (USA)] 3, 3', 5, 5' tetramethylbenzidine (TMB) substrate was added, 100 µL/well, and allowed to develop for 10 minutes. The reaction was quenched using 100 µL 1M H₃PO₄. The plates were read with a spectrophotometer set at 450 nm (nanometers).

Reactivity of serum in the six different sandwiches formed was evaluated. The following are the six sandwiches wherein the asterisks indicate which antibody in the sandwich was labeled with biotin:

1. 29C12 (capture) and 4C7*;
2. 4C7 (capture) and 26C3*;
3. 4C7 (capture) and 31G7*;
4. RB 20B3 (capture) and 4C7*;
5. RB 20B3 (capture) and 26C3*; and
6. RB 20B3 (capture) and 31G7*.

The capture antibody (29C12) for the first sandwich was made to EGFr purified from A431 cell lysate; the capture antibody (4C7) for the second and third sandwich assays was made to intact EGFr (A431 cells); whereas the capture antibody for the last three sandwiches (RB20B3) was made to a peptide encoded by a retinoblastoma gene and was used as a negative control to test for any anti-mouse activity in the NHS.

The results indicated that there was EGFr activity in NHS in sandwiches 2 and 3 but not in 1 nor in the three negative controls (sandwiches 4-6). The capture antibody in sandwich 1 is 29C12 which is a monoclonal antibody that does not recognize epitopes on the ectodomain of the EGFr. Therefore, the results indicate that the portion of the EGFr that was captured in the assay contained at least part of the ectodomain of the EGFr.

EXAMPLE 2Normal, Benign & Breast Cancer Serum Panel Test
in EGFr Ectodomain Bridge Immunoassay

A431 lysate (prepared according to the procedure detailed above) was diluted to yield calibrators with values ranging between 0.05 - 2 femtomole per milliliter (fmol/mL). Ten normal sera, ten benign breast sera (from Bioheme, Salt Lake City, Utah), and ten metastatic breast cancer sera [from Dr. Doug Tormey at the Clinical Center at the University of Wisconsin Center for Health Sciences at the Medical School, Department of Human Oncology, Madison, WI (USA)] were diluted 1:100 and 1:500 in assay buffer which comprised 50 mM TRIS, pH 7.4 with 1% BSA, 0.05% TWEEN-20, 0.01% DNS and 0.1% KATHON [5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one; Rohm & Haas, Philadelphia, PA (USA)]. Two hundred μ L of standard or diluted serum was added to Nunc star tubes coated with streptavidin by the coating procedure detailed above. Two hundred μ L of a conjugate solution comprising the monoclonal antibody 31G7 conjugated to FITC at 500 ng/mL and the monoclonal antibody 4C7 labeled with HRP at 500 ng/mL in the assay buffer was then added to each tube, and the reaction was incubated for 2 hours at room temperature. Two hundred μ L of a solution containing the biotinylated bridge receptor which is a mouse monoclonal raised against FITC (conjugated to KLH) in the assay buffer at 4 μ g/mL was added to each tube and allowed to incubate 2 hours at room temperature. Tubes were decanted and washed three times with Tris/TWEEN-20 wash buffer (50 mM Tris at pH 7.4 and 0.05% TWEEN-20). One mL of TMB substrate was added to each tube and developed for 15 minutes at room temperature. The reaction was quenched with 1 mL 1M H_3PO_4 . Tubes were read at 450 using a Chem-Stat tube reader [Ocean Scientific, Garden Grove, CA (USA)]. The concentration values for the captured EGFr ectodomain protein in fmol/mL were calculated for the serum samples using the A431 standard curve for which the values were calculated from the A431 calibrators using software accompanying the Chem-Stat tube reader.

As shown in Table 1 which tabulates the 1:500 values for the sera, which were within the range of the assay, at a cut-off of 0.9 fmol/mL, 60% of the cancer sera were positive and 0% of the normal and benign sera were positive. Further, as indicated at the bottom of Table 1, the mean for the cancer sera is 0.87 which is considerably higher than the means for the normal (mean is 0.38) and benign sera (mean is 0.59). Thus there is a higher concentration of the EGFr ectodomain protein captured in the bridge immunoassay by monoclonal antibodies, which recognize epitopes on the EGFr extracellular domain, in the cancer sera than in the normal and benign sera.

Table 1

	<u>Normal Sera</u>		<u>Benign Sera</u>		<u>Cancer Sera</u>	
	<u>Sample Nos.</u>	<u>fmol/mL</u>	<u>Sample Nos.</u>	<u>fmol/mL</u>	<u>Sample Nos.</u>	<u>fmol/mL</u>
	89	0.04	1	0.44	1	0.68
	90	0.55	2	0.18	2	0.94
	91	0.54	3	0.87	3	0.80
	92	0.08	4	0.56	4	0.94
	93	0.53	5	0.54	5	0.92
	94	0.12	6	0.77	6	0.53
	95	0.40	7	0.41	7	1.16
	96	0.54	9	0.64	8	0.90
	97	0.46	10	0.77	9	1.04
	98	0.11	11	0.70	10	0.82
	<u>Mean</u>	0.38		0.59		0.87
	<u>S.D.</u>	0.22		0.21		0.18

EXAMPLE 3

HPLC of Serum to Determine Approximate
Size Range of EGFr Portions

HPLC was performed to determine the approximate size range for the proteins from sera captured by the monoclonal antibodies that recognize epitopes on the ectodomain of EGFr as indicated in the above two examples. Serum samples were run over a DuPont Zorbax Gf-250 sizing column [DuPont Chem. Co., Wilmington, DE (USA)]. The mobile phase (buffer A) used was PBS (10 mM sodium phosphate at pH

7.4 with 0.1 M NaCl) with 0.05% TWEEN-20. The serum samples were run in the following order: 1) normal mouse serum [Sigma Chem. Co., St. Louis, MO (USA)] 2) normal human serum (from Bioheme) 3) metastatic breast cancer sera (from Dr. Tormey at The University of Wisconsin) and 4) normal mouse serum spiked with A431 lysate to yield a concentration of 62 fmol/mL. β -amylase (200,000), Albumin (66,000) and Carbonic Anhydrase (29,000) (markers from Sigma Chem., Co.) were run last as molecular weight standards. Two hundred μ L of each sample was injected onto the column. The column was washed for 20 minutes with buffer A between sample applications. 0.5 mL fractions (30 seconds) were collected starting at exactly 3 minutes post-injection for all of the serum samples. The resulting fractions were diluted 1:1 in assay buffer and tested according to the sandwich bridge immunoassay described in Example 2.

No positive immunoassay results were found for the mouse serum. Activity was found in fractions 12 and 13 of the normal and cancer samples which correspond to a molecular weight range from about 70,000 kd to about 120,000 kd. No activity was found in any of the other fractions. In the A431-spiked normal mouse serum, positive assay results were found in fractions 11-15 corresponding to a molecular weight range from about 60,000 kd to about 200,000 kd. The results show that the positive EGFr activity found in serum is produced by a protein and/or proteins in a molecular weight range encompassing that for the ectodomain of the EGFr, approximately 100 kd. Further, the results show that the molecular weight range for the protein and/or proteins found in the A431-spiked normal mouse serum, encompasses that for the EGFr in its entirety, that is, about 170 kd.

EXAMPLE 4

Western Blotting of EGFr Ectodomain Protein from Human Serum

To characterize an antigen identified by an appropriate antibody, a modification of the Western blot as

described by Towbin et al., PNAS (USA), 76:4350-4354 (1979) was used. Specifically, a 300 μ L sample of normal human serum was reacted with an anti-EGFr polyclonal antibody, EGFR #2 at a 1:300 concentration in 900 μ L PBS (10 mM sodium phosphate at 7.4 with 1M NaCl) overnight at 4°C. Immune complexes were precipitated by incubation with 75 μ L of a 2% Protein A-Sepharose (Sigma Chem. Co.) suspension for 2 hr at 4°C with end-over-end rotation. After being pelleted by centrifugation at 8,000 rpm for 5 min in a Beckman Microfuge 12 [Beckman Instruments, San Francisco, CA (USA)], the Protein A-Sepharose complexes were washed in one mL 100 mM NaCl, 10 mM Tris-HCl, pH 7.4, 0.2% NP-40 (Nonidet P-40; non-ionic detergent from Sigma Chem. Co., St. Louis, MO), followed by centrifugation. A subsequent wash was done using one mL 10 mM Tris-HCl, pH 7.4, 0.2% NP-40. After centrifugation, the immunoprecipitate was resuspended in 150 μ L SDS-PAGE sample buffer (0.0625 M Tris, pH 6.8, 3% SDS, 5% mercaptoethanol, 10% glycerol) and treated at 100°C for 5 min to solubilize the immune complexes.

Proteins were analyzed by SDS-PAGE as described by Laemmli, Nature, 227:680-685 (1970). Samples were loaded onto a 7.5% Laemmli SDS-polyacrylamide gel and separated by electrophoresis at 8 milliampere (mA) for approximately 16 hrs. Separated proteins were transferred to a nitrocellulose filter [Schleicher and Schuell, Kane, NH (USA)] in a Trans-blot transfer cell [BioRad Laboratories, Hercules, CA (USA)] using a 20 mM Tris, pH 8.2, 125 mM glycine buffer with 20% methanol at 25 volt (V) for 16 hrs. After transfer was complete, the nitrocellulose filter was blocked by a one hr incubation in 4% non-fat dry milk in PBS with 0.05% TWEEN-20 to prevent non-specific binding.

The MAb 31G7 at 10 μ g/mL in PBS-TWEEN-20 was reacted with the filter for 2 hrs at 37°C. After three 5-min washes in PBS-TWEEN-20, the filter was incubated in Vectastain goat anti-mouse (Vector Laboratories, Burlingame, CA) according to the manufacturer's directions, followed by incubation with the Vectastain ABC reagent (Vector Laboratories). A DAB [3, 3'-diaminobenzidine

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tetrahydrochloride (Sigma, St. Louis, MO) solution (10 mg in 50 mM Tris, pH 7.4, with 75 μ L 30% H_2O_2) was used to visualize the reaction.

A broad band with a molecular weight of about 100,000 daltons (reported in the literature as the approximate molecular weight for the ectodomain of EGFr) was seen in the human serum sample. The Western blot of immunoprecipitated human serum was repeated on a larger volume (1.2 mL) of a different normal serum sample; again a broad band with a molecular weight of about 100,000 daltons was seen, yet in this case the band contained approximately four times the protein seen in the first Western blot.

EXAMPLE 5

Western Blotting to Determine Specificity

Identical samples of normal human serum were immunoprecipitated with either EGFr #2 polyclonal antiserum or PEP-EGFr #1 polyclonal antiserum using the methods described in Example 4. The immunoprecipitated proteins were separated by SDS-PAGE and then transferred to a nitrocellulose filter which was probed with the MAb 31G7. The 100 kd protein present in serum was successfully precipitated by EGFr #2 antiserum that recognizes epitopes along the entire EGFr molecule; however, it was not precipitated by PEP-EGFR #1 antiserum that recognizes only the C-terminal end of the cytoplasmic domain of the EGFr.

EXAMPLE 6

Purification of EGFr Ectodomain Protein from Human Serum

The EGFr ectodomain protein was purified from human serum in order to confirm its identity through amino acid sequence data on the 20 amino acids located at the N-terminus. An EGFr affinity column was made by coupling 12.8 mg of MAb 31G7 to 3 ml of tresyl-activated agarose [Pierce, Rockford, IL (USA)] according to the manufacturer's instructions. Prior to use, the column (1.0 cm by 20 cm) was washed with 10 column volumes each of: (1) 1 M NaCl, (2) 50 mM triethylamine, pH 10.5, and (3) 50 mM sodium

citrate, pH 3.0 to remove uncoupled antibody. The column was then equilibrated with 10 column volumes of 20 mM HEPES, pH 7.5 containing 150 mM NaCl, 0.1% Triton X-100 and 10% glycerol (HTNG buffer).

5 One liter of pooled normal human serum [Biomedical Resources, Hatboro, PA (USA); lot # BM0325501] was diluted 1:1 in 20 mM HEPES, pH 7.5 buffer, with 150 mM NaCl and 0.1% Triton X-100 and filtered through a 0.45 micron filter. The serum was applied to the column at a flow rate of 30 ml per
10 hour over a period of several days at 4°C. Before elution, the column was washed with 10 column volumes of: (1) HTNG buffer, (2) 1 M NaCl, (3) distilled water, followed by (4) HTNG buffer. The EGFr ectodomain protein was eluted from the column in 5 column volumes of 50 mM triethylamine, pH
15 10.5. Twenty-five fractions were collected (0.6 ml each).

All fractions were assayed at a 1:40 dilution in a bridge immunoassay essentially as described in Example 2. The only deviation from the protocol described in Example 2 was the use of the monoclonal antibody 4C7 labeled with HRP
20 (using the NSBA cross-linking reagent rather than the SMCC cross-linking reagent) at 100 rather than 500 ng/mL. The concentration of EGFr ectodomain protein in fmol/ml was calculated in each fraction using a calibration curve. Fractions 3-25 were pooled and incubated with 1 ml Protein
25 G-agarose [Genex Corporation; Gathersburg, MD (USA)] for 1 hr to adsorb human IgG that may have been co-purified from the serum. The supernatant was then concentrated to approximately 500 µl in a Centricon 30 microconcentrator [Amicon, Danvers, MA (USA)].

30 The affinity-purified ectodomain protein was run on a 7.5% SDS-polyacrylamide reducing gel as described in Example 4. The proteins separated by electrophoresis were transferred to a ProBlott^R membrane [Applied Biosystems, Foster City, CA (USA)] using the method recommended by the
35 manufacturer. After transfer, the ProBlott membrane was stained for 30 sec in 0.1% Coomassie Blue R-250 in 40% methanol/1% acetic acid. A strongly staining band with a molecular weight of approximately 100,000 daltons (100 kd)

was cut out of the membrane. The band was sequenced at the Protein Structure Laboratory, School of Medicine, University of California, Davis, CA (USA) by subjecting the band to direct solid-phase Edman degradation on a 470 gas-phase sequencer [Applied Biosystems; Foster City, CA (USA)]. The PTH amino acids were analyzed using an H-P 35900C high pressure liquid chromatograph [Hewlett-Packard Company; Palo Alto, CA (USA)].

The sequence for the 20 N-terminal amino acids was reported to be LEEKKVHQGTSNKLTQLGTF. The sequence was substantially identical to the N-terminal sequence of EGFr as reported in Ullrich et al., Nature, 309: 418-425 (1984), the only difference being that there was a histidine at position 7 at which position Ullrich et al. indicated a cysteine.

A portion of the ProBlott membrane was analyzed by Western blotting using the MAb 31G7 followed by the reagents in the Vectastain ABC kit [Vector Laboratories; Burlingame, CA (USA)] as described in Example 4. A diffuse band was visualized at the 100,000 dalton position, as was seen in the human serum samples in Example 4.

Various modifications of the invention in addition to those shown and described herein will become apparent to those in the art from the foregoing description. Such modifications are intended to be within the scope of the appended claims. The references cited herein are hereby incorporated by reference.

CLAIMS

What we claim is:

1. A diagnostic/prognostic method for neoplastic disease comprising detecting and/or quantitating in a mammalian body fluid a portion of the epidermal growth factor receptor (EGFr) which comprises substantially the EGFr ectodomain and which has a molecular weight in the range of from about 90 kilodaltons (kd) to about 115 kd.
2. A diagnostic/prognostic method according to Claim 1 which comprises contacting a composition containing antibodies reactive with epitopes within said EGFr portion with a sample of the mammalian body fluid and determining whether said antibodies bind to an EGFr ectodomain protein in said sample.
3. A diagnostic/prognostic method according to Claims 1 or 2 wherein said molecular weight range is from about 95 kd to about 105 kd.
4. A diagnostic/prognostic method according to Claims 1 or 2 wherein said molecular weight range is about 100 kd.
5. A diagnostic/prognostic method according to Claim 1 wherein one end of the amino acid sequence of said EGFr portion is substantially identical in sequence to that reported for the N-terminus of the EGFr.
6. A diagnostic/prognostic method according to Claims 1-5 wherein the mammalian body fluid is a human body fluid selected from the group consisting of blood, plasma,

serum, sputum, ascites, semen, breast exudate, saliva, urine, cytosols, pleural effusions, bronchioalveolar lavages, bladder washes, amniotic fluid and cerebrospinal fluid.

5

7. A diagnostic/prognostic method according to Claims 1-6 wherein the neoplastic disease is a cancer selected from the group consisting of breast cancer; gynecologic cancer selected from the group consisting of ovarian, cervical, vaginal, endometrial, and vulval cancers; 10 gastrointestinal cancer selected from the group consisting of stomach, esophageal, and colon cancers; urinary cancer selected from the group consisting of bladder and kidney cancers; skin cancer; liver cancer; prostate cancer; 15 squamous cell carcinomas of the head and neck; and lung cancer.

8. A diagnostic/prognostic method according to Claims 1-7 which comprises the use of a bridge immunoassay 20 format which is a sandwich assay wherein an immunocomplex is formed in a liquid reaction between said EGFr portion and antibodies reactive therewith, wherein one of said antibodies is conjugated to a hapten and one of which is enzymatically labeled, and wherein a biotinylated bridge 25 antibody raised against said hapten is then added to the reaction mixture and links said immunocomplex to a solid phase that has been coated with avidin or streptavidin.

9. A diagnostic/prognostic method according to 30 Claims 1-7 in a competitive immunoassay format in which are used labeled proteins and/or polypeptides from the EGFr ectodomain and/or labeled anti-idiotypic antibodies to antibodies reactive with epitopes on the EGFr ectodomain.

35 10. A diagnostic/prognostic method for neoplastic disease comprising detecting and/or quantitating in mammalian tissue extracts a portion of the epidermal growth factor receptor (EGFr) which comprises substantially the

EGFr ectodomain and which has a molecular weight in the range of about 90 kilodaltons (kd) to about 115 kd.

11. A diagnostic/prognostic method according to
5 Claims 1-10 further comprising detecting and/or quantitating fragments which contain one or more epitopes of said EGFr portion.

12. A diagnostic/prognostic method according to
10 Claim 11 wherein said mammalian body fluid is serum or urine.

13. A test kit for assaying mammalian body fluids for a portion of the EGFr which comprises substantially the
15 EGFr ectodomain and which has a molecular weight in the range of from about 90 kd to about 115 kd, wherein said test kit comprises:

- a. antibodies reactive with said EGFr portion;
- and
- 20 b. a detection means.

14. A test kit according to Claim 13 further comprising labeled proteins and/or polypeptides from the EGFr ectodomain and/or labeled anti-idiotypic antibodies to
25 antibodies reactive with said EGFr portion.

15. A composition of matter comprising a substantially pure EGFr ectodomain protein isolated from one or more mammalian body fluids which has a molecular weight
30 in the range of from about 90 kd to about 115 kd.

16. A composition of matter according to Claim 15, further comprising substantially pure fragments of said EGFr ectodomain protein which fragments were also isolated from
35 one or more mammalian body fluids.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/04556

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12Q 1/00; G01N 33/556; C07K 3/00, 13/00, 15/00 17/00
US CL :530/350; 435/7.1; 436/501, 63

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350; 435/7.1; 436/501, 63

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
noneElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Medline, APS, dialog

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings Natl. Acad. Sci. USA, Volume 87, issued June 1990, P. A. Humphrey et al., "Anti-synthetic peptide antibody reacting at the fusion junction of deletion-mutant epidermal growth factor receptors in human glioblastoma", pages 4207-4211, see entire document.	1-16
Y	Cancer Research, volume 48, issued 15 April 1988, P. A. Humphrey et al., "Amplification and expression of the epidermal growth factor receptor gene in human glioma xenografts", pages 2231-2238, see entire document.	1-14
Y	Proc. R. Soc. Lond., Volume B226, W. J. Gullick et al., "The structure and function of the epidermal growth factor receptor studied by using antisynthetic peptide antibodies", pages 127-134, see entire document.	1-16
Y	Science, volume 224, issued 20 April 1984, W. Weber et al., "Production of an epidermal growth factor receptor-related protein", pages 294-297, see entire document.	1-14

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Z"	document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means		
"P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

20 AUGUST 1992

Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/04556

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US,A, 4,933,294 (Waterfield et al.) 12 June 1990, see entire document.	1-14
Y	Acta Endocrinologica, volume 117, Supplement 287, issued February 1988, W. Weber, "Truncated EGF REceptor: functional aspects of its secretion" page 47, see entire document.	1-14